Zeaxanthin and the Induction and Relaxation Kinetics of the Dissipation of Excess Excitation Energy in Leaves in 2% O₂, 0% CO₂

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ABSTRACT

The relationship between the carotenoid zeaxanthin, formed by violaxanthin de-epoxidation, and nonphotochemical fluorescence quenching (qNP) in the light was investigated in leaves of Glycine max during a transient from dark to light in 2% O₂, 0% CO₂ at 100 to 200 micromoles of photons per square meter per second. (a) Up to a qNP (which can vary between 0 and 1) of about 0.7, the zeaxanthin content of leaves was linearly correlated with qNP as well as with the rate constant for radiationless energy dissipation in the antenna chlorophyll (kD). Beyond this point, at very high degrees of fluorescence quenching, only kD was directly proportional to the zeaxanthin content. (b) The relationship between zeaxanthin and kD was qualitatively similar for the rapidly relaxing quenching induced in 2% O₂, 0% CO₂ at 200 micromoles of photons per square meter per second and for the sustained quenching induced by long-term exposure of Nerium oleander to drought in high light (B Demmig, K Winter, A Krüger, F-C Czygan [1988] Plant Physiol 87: 17–24). These findings suggest that the same dissipation process may be induced by very different treatments and that this particular dissipation process can have widely different relaxation kinetics. (c) A rapid induction of strong nonphotochemical fluorescence quenching within about 1 minute was observed exclusively in leaves which already contained a background level of zeaxanthin.

In leaves exposed to light in excess to that which is used in photosynthetic electron transport, the excess of excitation energy can be dissipated through radiationless dissipation in the photosynthetic system which results in nonphotochemical fluorescence quenching (4, 11, 13, 14, 19). We have previously reported (6, 7) that one component of nonphotochemical fluorescence quenching which relaxes relatively slowly upon darkening of leaves shows a correlation with the level of the carotenoid zeaxanthin, formed by violaxanthin de-epoxidation in the thylakoid membranes (10, 21). This type of quenching could be induced either by transfer of leaves to an atmosphere containing no CO₂ and only 2% O₂ (6), in which both photosynthesis and photorespiration are largely inhibited, or by a long-term water stress treatment of Nerium oleander in high light (7).

Recently, however, we have reported (8) that zeaxanthin is correlated with all of the dissipative nonphotochemical quenching in high light in air, i.e. high-energy-state quenching which relaxes rapidly. In the present study we investigate the relationship between zeaxanthin and nonphotochemical fluorescence quenching directly in the light in 2% O₂, 0% CO₂, and compare the ratio between kD and zeaxanthin in the light in this treatment with the ratio determined for the extremely slowly relaxing type of response in water-stressed N. oleander.

Previous findings on the induction and relaxation kinetics for violaxanthin de-epoxidation and zeaxanthin epoxidation (6, 9, 10, 17) versus those for high-energy-state quenching (5, 11, 13–15) do not seem to be consistent with a function of zeaxanthin as a fluorescence quencher. In the present study we therefore compare the induction kinetics for this type of fluorescence quenching in zeaxanthin-free leaves with those in leaves containing a background level of zeaxanthin and show that a very rapidly developing component of nonphotochemical fluorescence quenching was observed exclusively in zeaxanthin-containing leaves.

MATERIALS AND METHODS

Plant Material

The mangrove Rhizophora mangle L. was grown from vegetative propagules collected in Venezuela. Plants were cultivated in nutrient solution containing artificial sea salt (Sigma, St Louis, MO) equivalent to 10% seawater, in a temperature-controlled, naturally lit glasshouse at approximately 30°C/22°C (day/night) and 60/80% RH. The experimental leaves developed during fall and winter with mostly cloudy skies (up to 300 μmol photons m⁻² s⁻¹). Populus balsamifera L. was grown in 25-L pots filled with garden soil outdoors in the Würzburg Botanical garden. The leaves used

1 Abbreviations: kD, rate constant for radiationless energy dissipation in the antenna Chl; DW, dry weight; F₀, yield of instantaneous Chl fluorescence; Fₚₚ, yield of maximum Chl fluorescence, induced in a pulse of saturating light; Fᵥ, yield of variable fluorescence, induced in a pulse of saturating light; Fᵥ', variable component of steady state fluorescence emission in the light; Q, primary electron acceptor of PSII; qNP, quenching coefficient for nonphotochemical fluorescence quenching.
here had developed during the summer of 1987. Plants were kept fertilized and well-watered. Leaves of Hedera helix L. were obtained from a natural population growing at a shaded site in the Würzburg Botanical garden. Monstera deliciosa Liebm. was kept in a glasshouse at 20 to 30 μmol photons m⁻² s⁻¹. Glycine max L. Merril (soybean) was grown in growth cabinets at a PFD of 400 μmol m⁻² s⁻¹ under a 12 h photoperiod (25°C days/18°C night). For the experimental treatments the plants were first transferred to the glasshouse for a period of 3 d, where they were exposed to natural light during the fall of 1987 with mostly cloudy skies.

Experimental Treatment of Leaves

Attached intact leaves were sealed into a ventilated, temperature-controlled gas exchange chamber and were exposed to the desired PFD in a gas stream of either ambient air (21% O₂, 0.034% CO₂) or a CO₂-free mixture of 2% O₂ and 98% N₂. Leaf temperature was kept constant at 25°C, and the water vapor pressure difference between the leaf and the surrounding atmosphere was kept in the range 0.5 to 1 kPa. For continuous recording of Chl a fluorescence a double-sided glass and aluminum chamber (exposed leaf area: 13.5 cm²) was used (20) and illumination was provided by an Osram Power Star HQI-R 250 W/N Dulap lamp (Osram, München, FRG). For the treatment of leaves for pigment analysis, a 600-mL chamber made of glass and brass was used and illumination was provided by a metal halide lamp (HRI-T 1000 W/D; Radium Elektrizitäts-Ges., Wipperfürth, FRG). IR radiation was reduced using a 6 mm thick heat reflecting glass (Tempax; Schott, Mainz, FRG). The time courses of fluorescence quenching proved to be highly reproducible among leaves of the same age of a given species. Therefore, leaf discs for pigment analysis were sampled from larger exposed leaf areas in separate experiments. For removal of leaf discs (4–10 cm²), the chamber was briefly opened, and a leaf disc was punched and immediately immersed in a Dewar containing liquid N₂. The leaf disc was darkened for no more than 3 s before metabolism was stopped in liquid N₂. The DW was determined on corresponding leaf discs.

Fluorescence and Pigment Analysis

Chl a fluorescence was continuously recorded in illuminated leaves using a pulse amplitude modulation fluorometer (model PAM 101; H. Walz, Effeltrich, FRG) (6). The leaf was illuminated with photosynthetically active radiation at an angle of 60° from one side and fluorescence was excited and detected by a fiber optic probe touching the upper surface of the leaf at 60° and fixed in this position on the other side. Leaves were taken from the growth cabinet or glasshouse at the end of the photoperiod and were sealed into the gas exchange chamber. Leaves were then held in darkness overnight for 12 h, after which the control level of F₀ fluorescence at open PSIII traps and F₄₅ fluorescence at closed PSIII traps were determined (cf. refs. 4, 7). The corresponding F₀ value was used as the reference value (F₄₅control) for the determination of the quenching coefficient qNp. The composition of the gas passed over the leaves was either ambient air or a CO₂-free mixture of 2% O₂, 98% N₂. During the illumination with photosynthetically active radiation, the weak measuring beam of the PAM system was used to excite steady state fluorescence, composed of F₀ and a component F₄₅', which comes from the PSII centers that are already closed at that level of photosynthetically active radiation. A saturation pulse given thereafter removes photochemical quenching related to photosynthetic electron transport and thus allows one to determine the remaining nonphotochemical quenching which lowers PSII fluorescence predominantly by radiationless energy dissipation (but also potentially by other kinds of fluorescence quenching such as, e.g. transfer of excitation energy to PSI). The quenching coefficient qNp is 1 − F₄₅/F₄₅control, where F₄₅ is the variable fluorescence in a saturation pulse in the light. We calculated the k₀ (8) according to the simple model by Kitajima and Butler from k₀=1/F₄₅ − 1 (12; cf. also 1, 4).

During the water stress treatment of Nerium oleander (7), F₀ and F₄₅ were determined at the end of the 12 h dark period for up to 14 d following the termination of watering. The leaves had developed and were kept at high PFD (800–1000 μmol m⁻² s⁻¹) during the 12 h light period throughout the treatment. The variable fluorescence, Fᵥ, at the end of the 12 h dark period during the treatment was compared with the variable fluorescence, Fᵥcontrol, at the end of the 12 h dark period in a well-watered control leaf (7). The expression 1 − Fᵥ/Fᵥcontrol in this experiment corresponds to the quenching coefficient for nonphotochemical fluorescence quenching during PAR illumination, qNp.

Pigments were analyzed quantitatively after separation by TLC (3, 18). Further procedures and calculations were as described previously (6, 8).

RESULTS

Relationship between Zeaxanthin Content, qNp, and k₀

Figure 1A shows Chl a fluorescence recorded continuously in the light during a dark/light transition in a leaf of Glycine max. At various time points during the transient, pulses of saturating light were given to obtain F₄₅, and consequently the degree of nonphotochemical fluorescence quenching. The leaf was kept in 2% O₂, 0% CO₂ to promote a strong increase in the type of nonphotochemical fluorescence quenching reflecting radiationless dissipation of excitation energy (4). From the decrease in F₄₅, the rate constant for radiationless dissipation in the antenna Chl, k₀, (Fig. 1B) was calculated after Kitajima and Butler (12; cf. also 1, 4). The time course of the increase in k₀ and that of the increase in zeaxanthin content were very similar (Fig. 1B).

For the same experiment, Fᵥ and Fᵥ/F₄₅ (Fig. 2A), the quenching coefficient qNp [= 1 − (Fᵥ/Fᵥcontrol)] (Fig. 2B), and the rate constant for radiationless energy dissipation, k₀ (Fig. 2C) are shown as a function of leaf zeaxanthin content. The increase in zeaxanthin was proportional to an increase in qNp up to approximately 0.7. At higher degrees of quenching, qNp was no longer linearly related to zeaxanthin. By contrast, the rate constant k₀ was linearly related to the zeaxanthin content over the entire range of quenching.

Figure 3 shows data for Nerium oleander from a previous paper (7) plotted in a manner similar to the data presented in Figure 2. These N. oleander leaves were subjected to a combination of high light and water stress which had developed
gradually over a period of 10 d. The treatment resulted in a sustained reduction in PSII photochemical efficiency $F_v/F_M$. The data points shown here represent samples taken at the end of the 12 h dark period as opposed to the above measurements in the light. The relationship between the fluorescence parameters and zeaxanthin shown in Figures 2 and 3 is strikingly similar. The magnitude of the increase in $k_D$ from 10 or 20 to 50 or 60 was similar in these completely different treatments. Variable fluorescence and $1 - F_v/F_{\text{control}}$ (which corresponds formally to $q_{NP}$) do not show linear relationships with zeaxanthin at high degrees of quenching in either case.

**Relaxation Kinetics of Nonphotochemical Quenching upon Darkening of Leaves**

Dark relaxation kinetics of fluorescence quenching induced in 2% $O_2$, 0% $CO_2$, and 120 $\mu$mol photons m$^{-2}$ s$^{-1}$ were studied in $G.\ max$ and in a mangrove, *Rhizophora mangle* (Fig. 4). The mangrove is another example of a sclerophyllous leaf, and it exhibits similar responses of photochemistry to a combination of high light and very negative leaf water potentials as *N. oleander* (2). Figure 4 shows, first, the decrease of $F_M$ in the light in leaves subjected to a dark/light transition in 2% $O_2$, 0% $CO_2$. At various time points samples were removed from the leaves and transferred to darkness. In both $G.\ max$ (Fig. 4A) and *R. mangle* (Fig. 4B) the portion of the quenching of $F_M$ which relaxed within e.g. 2 min (5) became increasingly smaller after prolonged exposure to 120 $\mu$mol photons m$^{-2}$ s$^{-1}$ in 2% $O_2$, 0% $CO_2$.

**Induction Kinetics of Nonphotochemical Fluorescence Quenching upon Exposure to 2% $O_2$, 0% $CO_2$**

For the experiments shown in Figures 5 and 6, nonphotochemical fluorescence quenching was induced with no change
Figure 3. Relationship between (A) $F_v/F_m$ and $F_v$, (B) fluorescence quenching still present after 12 h dark, $1 - F_v/F_{v_{control}}$, and (C) the rate constant for radiationless energy dissipation (heat dissipation) $k_0$, and the zeaxanthin content in leaves of *N. oleander* subjected to slowly developing water stress (0–14 d) at a PFD of 800 μmol m$^{-2}$ s$^{-1}$ (data are from ref. 7).

Periods of 5 min in all three leaves (Fig. 5, A, B, and C), indicating that the reduction state of Q (cf. refs. 15, 16, 20) had increased strongly initially and then decreased in parallel with the development of nonphotochemical quenching. After 30 min in 2% O$_2$ and 0% CO$_2$, air was passed over the leaves for an additional 60 min after which maximum fluorescence was determined in a saturation pulse in the light that had reached a level which was similarly high to the one determined prior to the first treatment with 2% O$_2$, 0% CO$_2$ (Fig. 5, A, B, and C). When the composition of the gas stream at this point was changed to 2% O$_2$, 0% CO$_2$ for a second time, $F_m$ decreased much more rapidly than during the first treatment and steady state fluorescence ($F_{v'} + F_o$) never attained such high values as in the first treatment (Fig. 5).

Figure 4. Time course of the decrease in maximum fluorescence yield ($F_m$) in leaves of (A) *G. max* and (B) *R. mangle*, exposed to a PFD of 120 μmol m$^{-2}$ s$^{-1}$ in 2% O$_2$, 0% CO$_2$. Leaves of *G. max* had been grown and treated as described in the legend of Figure 1. $F_m$ was determined in a pulse of saturating light given during illumination with photosynthetically active radiation (open circles), as well as in pulses of saturating light given after 2 min (closed circles), 5 min (closed triangles), and 10 min (closed squares) in discs which were removed from the treated leaves and kept in darkness.
Figure 5. Time course of changes in Chl a fluorescence recorded continuously in the light in leaves of (A) *M. deliciosa*, (B) *H. helix*, and (C) *P. balsamifera*, suddenly exposed to 2% O<sub>2</sub>, 0% CO<sub>2</sub> at a PFD of 60 to 100 μmol m<sup>-2</sup> s<sup>-1</sup>. The leaves were sealed into a gas exchange chamber and then exposed to air and PFDs of 10, 20, 30, and 50 μmol m<sup>-2</sup> s<sup>-1</sup> for 15, 10, 10, and 5 min, respectively, to avoid any sudden large increases in PFD. Then the PFD was increased to 60 to 100 μmol m<sup>-2</sup> s<sup>-1</sup>. The first saturation pulse which was shown given after 60 min at these PFDs in air. Then the air was replaced (treatment 1) by a gas stream of 2% O<sub>2</sub>, 0% CO<sub>2</sub> for 30 min. After that, air was again passed over the leaves for 60 min and maximum fluorescence was determined again. This was followed by a second treatment (treatment 2) in 2% O<sub>2</sub>, 0% CO<sub>2</sub>. During the treatments, steady state fluorescence was recorded continuously and pulses of saturating light were given at certain time points. In the insets is shown the zeaxanthin content of leaves determined prior to the first and the second treatment with 2% O<sub>2</sub>, 0% CO<sub>2</sub>, i.e. at the end of the 60 min recovery period in air.

mined for a corresponding leaf after 30 min in 2% O<sub>2</sub>, 0% CO<sub>2</sub> (not shown). In the third treatment F<sub>M</sub> fell almost instantaneously to less than 50% of the control value. During the 60 min period at low light in air, F<sub>M</sub> recovered almost completely whereas the zeaxanthin level did not return to the control level. Thus, under these control conditions there was clearly no correlation between zeaxanthin and fluorescence quenching.

DISCUSSION

Relationship between Zeaxanthin, q<sub>np</sub>, and k<sub>0</sub>

The correlation between zeaxanthin content and nonphotochemical fluorescence quenching in the light during dark/light transients in 2% O<sub>2</sub>, 0% CO<sub>2</sub> in low light (Fig. 2) was similar to that observed previously (8) for the type of fluorescence quenching which occurs in air in the light-saturated range of photosynthetic CO<sub>2</sub> uptake, i.e. the quenching which is related to the high-energy state of the thylakoid membrane (11, 15, 19). Under the present conditions, all nonphotochemical fluorescence quenching in the light showed a linear relationship with the zeaxanthin content (Fig. 2), whereas in air there was an additional type of fluorescence quenching which occurred at light levels below the light-saturation of net CO<sub>2</sub> uptake (5, 8). Similar to this previous study (8), q<sub>np</sub> increased less than the zeaxanthin content at high degrees of quenching, whereas k<sub>0</sub> was directly proportional to the zeaxanthin content at all times.

When we first reported a correlation between zeaxanthin and energy dissipation (6), we used the same conditions as are used here (2% O<sub>2</sub>, 0% CO<sub>2</sub>) to induce strong energy dissipation. In this previous study, however, both fluorescence and zeaxanthin were determined in samples predarkened for 5 min and there was much less fluorescence quenching for a given zeaxanthin content than in water-stressed *Nerium oleander* leaves (Fig. 3). The greater extent of fluorescence quenching in the light for a given zeaxanthin content (Fig. 2), however, is very similar to that found in *N. oleander* for which fluorescence and zeaxanthin were determined after 12 h of darkness (Fig. 3). The similarity of the relationship between zeaxanthin and F<sub>v</sub>, F<sub>v</sub>/F<sub>M</sub>, q<sub>np</sub>, and k<sub>0</sub> measured in the light during a dark/light transient (Fig. 2) and for sustained fluorescence quenching in water-stressed *N. oleander* leaves (Fig. 3), suggests the same underlying mechanism for radiationless energy dissipation. Since the relaxation of fluorescence quenching was within minutes in the former case (Fig. 2) but within days in the latter case (7), one would have to assume that the same process can exhibit very different relaxation kinetics. This is consistent with the observation that the rapidity of the relaxation of nonphotochemical fluorescence quenching decreased with increasing length of exposure (Fig. 4). Thus, the radiationless dissipation process induced within minutes and relaxing within minutes (Figs. 2 and 4) and that induced over days and relaxing over days (7) may have the same nature. The fact that a very similar relationship between zeaxanthin and k<sub>0</sub> exists in all of these cases is consistent with the hypothesis that zeaxanthin mediates this radiationless dissipation process. However, one would have to postulate an additional level of control (8) which, after short-term expo-
sure, allows the relaxation of fluorescence to be more rapid than the removal of zeaxanthin.

**Relationship between Zeaxanthin and a Rapidly Developing Component of Nonphotochemical Quenching**

In leaves which contained no zeaxanthin, the rapid component of fluorescence quenching was not observed (Figs. 1, 5, and 6). Instead, the kinetics of fluorescence quenching matched the kinetics of zeaxanthin synthesis in the xanthophyll cycle. In pretreated leaves which contained a background level of zeaxanthin, a rapid component of fluorescence quenching, lowering Fm by more than 50%, did occur within about 1 min (Figs. 5 and 6). This is more rapid than the enzymic conversion of violaxanthin to zeaxanthin (6, 9, 17). Since this rapidly developing component of fluorescence quenching was observed exclusively in leaves with a large background level of zeaxanthin, even this component of fluorescence quenching may be related to the presence of zeaxanthin.

The observation of high maximum fluorescence yield (Fm) in limiting light (100 μmol photons m⁻² s⁻¹ in air) in leaves which contained a large background level of zeaxanthin (Figs. 5 and 6) suggests either (a) that zeaxanthin does not act as a quencher of fluorescence or (b) that zeaxanthin, once synthesized, must be rendered effective (as a quencher of fluorescence) by some additional condition created by an excess of light. This additional condition could be the high-energy-state of the thylakoid membrane, possibly leading to a closer association between zeaxanthin and Chl molecules in the antennae complexes. If this was the case, the activity of zeaxanthin would be controlled, first, biochemically via the enzymic conversion of violaxanthin to zeaxanthin, which is in turn controlled by membrane energization (ΔpH and a redox component), and second, more directly by membrane energization in and by itself. Such an additional control mechanism would have the effect that even a fluorescence quenching mediated by zeaxanthin would exhibit induction and relaxation kinetics which are more rapid than the formation and removal of zeaxanthin.

The results presented here show that the capacity of leaves for the rapid development of strong nonphotochemical fluorescence quenching is closely related to the status of the photochemical apparatus of PSII prior to the exposure to excess light. There is a relationship between the presence of zeaxanthin in leaves and their capacity for an almost instantaneous increase in the heat dissipation activity under conditions where light becomes excessive in the absence of terminal electron acceptors.

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